1619 Rec'd PCT/PTO 28 JAN 2002

FORM PTO-1390 (REV 11-2000)	U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 1721-47
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S APPLICATION NO (If known, see 37 C F R. 1 5) 10/048116
INTERNATIONAL APPLICATION NO. **PCT/FR00/02193**	INTERNATIONAL FILING DATE **28 July 2000**	PRIORITY DATE CLAIMED **29 July 1999**

TITLE OF INVENTION
RECOMBINANT PROTEINS AND MOLECULAR COMPLEXES DERIVED FROM THESE PROTEINS, ANALOGOUS TO MOLECULES INVOLVED IN IMMUNE RESPONSES

APPLICANT(S) FOR DO/EO/US
GLAICHENHAUS et al

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The U.S. has been elected by the expiration of 19 months from the priority date (Article 31).
5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has **NOT** expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☒ A English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 To 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☐ Other items or information.

531 Rec'd PCT/PT 28 JAN 2002

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
Unknown 10/048116	PCT/FR00/02193	1721-47

21. ☒ The following fees are submitted:

	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5):	
-- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1040.00	
-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $890.00	
-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO $740.00	
-- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $710.00	
-- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).	$ 130.00

CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE		
Total Claims	11	-20 =	0	X	$18.00	$ 0.00
Independent Claims	1	-3 =	0	X	$84.00	0.00
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)					$280.00	$ 0.00

TOTAL OF ABOVE CALCULATIONS =	$ **1020.00**
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.	0.00
SUBTOTAL =	$ **1020.00**
Processing fee of $130.00, for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)). +	0.00
TOTAL NATIONAL FEE =	$ **1020.00**
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). **$40.00** per property +	$ 0.00
Fee for Petition to Revive Unintentionally Abandoned Application ($1280.00 – Small Entity = $640.00)	$ 0.00
TOTAL FEES ENCLOSED =	$ **1020.00**
Amount to be: refunded	$
Charged	$

a. ☒ A check in the amount of $1020.00 to cover the above fees is enclosed.
b. ☐ Please charge my Deposit Account No. 14-1140 in the amount of $_____ to cover the above fees. A duplicate copy of this form is enclosed.
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.
d. ☒ The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application.

NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

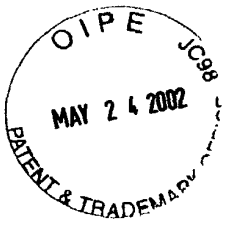
NIXON & VANDERHYE P.C.
1100 North Glebe Road, 8th Floor
Arlington, Virginia 22201-4714
Telephone: (703) 816-4000

B. J. Sadoff
SIGNATURE

B. J. Sadoff
NAME

36,663 **January 28, 2002**
REGISTRATION NUMBER Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE 24 MAY 2002



In re Patent Application of

GLAICHENHAUS et al

Serial No. 10/048,116

Filed: January 28, 2002

Atty. Ref.: 1721-47

Group:

Examiner:

For: RECOMBINANT PROTEINS AND MOLECULAR COMPLEXES DERIVED FROM THESE PROTEINS, ANALOGOUS TO MOLECULES INVOLVED IN IMMUNE RESPONSES

* * * * * * * * * * *

May 24, 2002

Honorable Assistant Commissioner of Patents
Washington, DC 20231

Sir:

STATEMENT

The attached paper and computer readable copies of the Sequence Listing are the same. No new matter has been added.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____

B. J. Sadoff
Reg. No. 36,663

1100 North Glebe Road, 8th Floor
Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

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10/048116
Rec'd PCT/PTO 24 MAY 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

GLAICHENHAUS et al — Atty. Ref.: 1721-47

Serial No. 10/048,116 — Group:

Filed: January 28, 2002 — Examiner:

For: RECOMBINANT PROTEINS AND MOLECULAR COMPLEXES DERIVED FROM THESE PROTEINS, ANALOGOUS TO MOLECULES INVOLVED IN IMMUNE RESPONSES

* * * * * * * * * * *

May 24, 2002

Honorable Assistant Commissioner of Patents
Washington, DC 20231

Sir:

AMENDMENT

Responsive to the Notification dated April 10, 2002, entry and consideration of the following amendments and remarks are requested.

IN THE SPECIFICATION:

Amend the specification as follows:

Insert the attached Sequence Listing after the claims pages.

REMARKS

Reconsideration is requested.

Responsive to the Notification dated April 10, 2002, the applicants submit that the requisite Declaration was filed February 27, 2002. A copy of the previously filed Declaration and the undersigned's cover sheet which was filed therewith on February 27, 2002 is attached for the convenience of the Office. A copy of the undersigned's

post card receipt from the filing of February 27, 2002 is attached as evidence the same was received by the Patent Office on February 27, 2002.

A copy of the Notification dated April 10, 2002 is attached.

The specification has been amended to include the attached Sequence Listing. The attached paper and computer readable copies of the Sequence Listing are the same. No new matter has been added. A separate Statement to this effect is attached.

An early and favorable Action on the merits is requested.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____

B. J. Sadoff
Reg. No. 36,663

1100 North Glebe Road, 8th Floor
Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

10/048116
531 Rec'd PCT/PTO 28 JAN 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

GLAICHENHAUS et al

Atty. Ref.: **1721-47**

Serial No. **Unknown**

Group:

National Phase of: **PCT/FR00/02193**
International Filing Date: **28 July 2000**

Filed: **Herewith**

Examiner:

For: **RECOMBINANT PROTEINS AND MOLECULAR COMPLEXES DERIVED FROM THESE PROTEINS, ANALOGOUS TO MOLECULES INVOLVED IN IMMUNE RESPONSES**

* * * * * * * * * * *

January 28, 2002

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

PRELIMINARY AMENDMENT

Prior to calculation of the filing fee and in order to place the above identified application in better condition for examination, please amend as follows:

IN THE SPECIFICATION

Page 1, after the title insert the following:

-- This application is the US national phase of international application PCT/FR00/02193 filed July 28, 2000 which designated the U.S. --.

IN THE CLAIMS (AS AMENDED)

Please substitute the following amended claims for corresponding claims previously presented. A copy of the amended claims showing current revisions is attached.

4. (Amended) Nucleotide sequences having a reading frame corresponding to all or part of a protein according to claim 1.

7. (Amended) Use of proteins according to claim 2, for counting and/or purifying the T lymphocytes that react with a given antigen and for characterizing the phenotype of these cells.

10. (Amended) Use according to claim 2, for the purification and/or enrichment of specific T lymphocytes of a given antigen, either from cell cultures, or from samples taken from a patient.

REMARKS

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached page is captioned "**Version with markings to show changes made**."

The above amendments are made to place the claims in a more traditional format.

The above amendments are based on the claims as amended in the International phase of the PCT application. A two page translation of a set of 11 claims is attached and should be the basis for the above amendments and the subject of the initial examination, unless the application is further amended in subsequently submitted papers. An English translation of he originally published application and set of 16 numbered claims is also attached, for completeness and in compliance with the rules and statute. An English translation of an Abstract is

also attached, as a separate sheet for insertion in the application in a manner deemed appropriate by the Patent Office.

The present filing is submitted to meet the requirements of 35 U.S.C. § 371 but for the submission of an executed Declaration. The Office is requested to contact the undersigned if anything further is required in this regard.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____

B. J. Sadoff
Reg. No. **36,663**

BJS:lmy

1100 North Glebe Road, 8th Floor
Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

4. (Amended) Nucleotide sequences having a reading frame corresponding to all or part of a protein according to [any one of claims 1 to 3] claim 1.

7. (Amended) Use of proteins according to claim 2 [or 3], for counting and/or purifying the T lymphocytes that react with a given antigen and for characterizing the phenotype of these cells.

10. (Amended) Use according to claim 2 [or 3], for the purification and/or enrichment of specific T lymphocytes of a given antigen, either from cell cultures, or from samples taken from a patient.

10/048116
531 Rec'd PCT/PT 28 JAN 2002

Recombinant proteins and molecular complexes derived from these proteins, analogous to molecules involved in immune responses.

The invention relates to recombinant proteins, and to molecular complexes derived from these proteins, analogous to molecules involved in immune responses.

It also relates to a method for producing such molecules and of such complexes, as well as their uses, in particular for diagnosis and in therapy.

It is known that molecules encoded by the Major Histocompatibility Complex (MHC) have a major role in an immune response.

These molecules are made up of two polypeptide chains: the heavy chain, and the light chain.

The molecules of the MHC are expressed on the surface of the presenting cells (dendritic cells, B lymphocytes, macrophages) in the form of molecular complexes with antigenic peptides, which are in turn derived from extracellular or intracellular proteins.

Recognition of these peptide/MHC complexes by a specific receptor expressed on the surface of the T lymphocytes is at the origin of any cell-mediated immune response.

The MHC molecules belong to two separate classes: those of class I, which are recognized by $CD8^+$ T lymphocytes (cytotoxic T cells) and those of class II which are recognized by $CD4^+$ T lymphocytes (helper T cells).

In order to be able to be used as probes for counting and measuring the frequency of specific T lymphocytes of a given antigen, such molecules and complexes have to be produced in soluble form. These same soluble molecules and complexes can be used for modulating the immune responses.

The possibility of using soluble MHC molecules for detecting $CD8^+$ T lymphocytes was first demonstrated by Altman et al. in 1996 (1). Since then, many teams have used this strategy for counting and characterizing the phenotype of $CD8^+$ T lymphocytes reacting with viral or bacterial peptides or peptides derived from tumour antigens. However, the application of this strategy for the detection of $CD4^+$ T lymphocytes has proved problematic.

In the majority of works published to date, bacterial expression systems have been used for producing class I MHC molecules. After incubation of these molecules with antigenic peptides, the peptide/MHC complexes were purified and obtained in the form of tetramers after incubation with streptavidin. This last stage is made possible by addition, to the carboxy-terminal end of the MHC heavy chain, of a recognition site for the BirA enzyme, a bacterial enzyme that is capable of catalysing the addition of a biotin molecule. Other teams chose to produce dimers of class I MHC molecules by using the skeleton of an antibody. In this case the MHC heavy chain was bound to the heavy chain of an immunoglobulin (abbreviated to Ig) and β-2-microglobulin was bound to the light chain. As the Fc regions of the heavy chains link together by means of sulphide bridges, the molecules produced are dimers of MHC molecules.

For technical reasons, the preparation of molecular probes that bind selectively to the $CD4^+$ T lymphocytes proved much more difficult, probably because of the intrinsic instability of the class II MHC molecules.

Tetramers of class II molecules bound to an antigenic peptide, or dimers of these molecules obtained using the skeleton of an antibody, have been produced.

The problem of the stability and affinity of the receptors of $CD4^+$ T lymphocytes for their ligand is solved, according to the invention, by employing constructs ensuring

the formation of dimers giving multivalent complexes owing to the use of molecules having several binding sites for certain regions of the dimers.

Such constructs can be envisaged both for class I MHC molecules and for those of class II.

Advantageously, the said constructs are sufficiently stable for use as molecular probes, thus opening up a wide field of application.

These constructs can also be used for obtaining analogues of T cell receptors capable of specifically recognizing such molecules.

The invention therefore aims to supply recombinant molecules and corresponding recombinant complexes, in which these molecules are bound to antigenic peptides, of great stability and with high affinity for their ligand.

Another aim is their production in host cells with the aid of suitable expression vectors.

A further aim of the invention relates to immunological applications of these complexes as molecular probes.

The soluble recombinant proteins according to the invention are constituted, as a minimum, from a dimer, itself formed from α and β chains of class I or II MHC molecules.

Other soluble recombinant proteins according to the invention are constituted, as a minimum, from a dimer, itself formed from two proteins each of which is made up of the whole or a fused part of alpha and beta chains of class I or II MHC molecules.

These dimers are characterized in that they have, at the carboxy terminal end of one or both chains, the whole or part of an Fc region of an immunoglobulin.

"Part of an Fc region" denotes a fragment corresponding to a natural fragment, or one modified relative to the said natural fragment, by substitution and/or by deletion and/or

by mutation, but capable of binding to a protein possessing binding sites for the Fc region, such as protein A or protein G.

The term "capable of binding" is illustrated by Example 1C.

The Fc region corresponds more particularly to the whole or part of the CH_2 and/or CH_3 domain. This domain can be modified relative to the natural domain, but must be capable, in accordance with the invention, of binding to a protein of the protein A or G type possessing several binding sites for the Fc region of an Ig.

The immunoglobulin having the constant region mentioned above can be an IgG, especially the isotypes IgG1, IgG2a, IgG2b, IgG3, an IgM, and IgA, and IgD or an IgE.

The proteins of the invention are more particularly characterized in that they comprise all or part of the α or β chains of the MHC molecules.

Advantageously, the α and β chains constituting the dimer contain leucine zippers, which promotes their pairing.

Such leucine zippers are described for example by Scott et al. (2) or Kalandadze et al. (3).

The invention relates in particular to recombinant molecules bound together as several dimers and particularly as tetramers and quite especially as octamers.

The said recombinant molecules are complexed with a natural or artificial protein comprising several binding sites for the constant regions of the immunoglobulins and thus permitting the creation of multimers from dimers. As an example protein A which is commonly isolated from *Staphylococcus aureus*, or protein G from *Streptococcus* (group C), or receptor multimers from the Fc regions obtained by genetic recombination can be mentioned.

The recombinant molecules as defined above, complexed to antigenic peptides, constitute MHC analogues. These are soluble recombinant proteins, characterized in that they are bound covalently or non-covalently to an antigenic peptide. The invention relates to the said complexes, characterized in that they have, at the $-NH_2$ end of the β chain, an antigenic peptide that is fixed by means of a flexible arm. This arm can be of a variable length and makes it possible to locate the antigenic peptide in the groove formed by the or each dimer. Fixations of this kind are described for example by Kozono et al. (4) and (5).

The molecules defined above are preferably obtained by the techniques described in textbooks of molecular biology for the preparation of recombinant genes and their expression in eukaryotic or prokaryotic cells. Reference should be made for example to the works of Sambrook et al. (6) or of Ausubel et al. (7).

The nucleotide sequences of the invention possess a reading frame corresponding to the whole or part of a protein as defined above.

The sequences coding for the recombinant fragments constituting the molecules defined above are introduced into expression vectors. Generally as many expression vectors as fragments are used. However, it is also possible, as a variant, to use an identical vector for several fragments. Plasmids and especially plasmids possessing a selection marker will be used advantageously as expression vectors. Satisfactory expression results have thus been obtained with plasmids that are able to replicate in bacteria and have, as selection marker, an antibiotic resistance gene.

The promoters will be selected so as to permit expression of the recombinant gene in the expression system used. As an example the promoter recognized by the polymerase of the T4 bacteriophage or, when using Drosophila

cells as the expression system, the promoter of the metallothionein gene may be mentioned.

As eukaryotic expression systems, we may mention the recombinant baculovirus systems in insect cells, Drosophila cells, hamster cells (CHO line) and monkey cells (COS line). It is also possible to effect expression in yeast cells.

Bacteria are widely used, in particular *E. coli*, as prokaryotic expression systems.

The recombinant molecules produced are purified on immunoaffinity columns, especially with monoclonal or polyclonal antibodies specific to the molecules of interest, or with supporting materials such as beads, especially agarose beads.

Other purification protocols can be envisaged. In particular, for example when the molecules to be purified have 6 consecutive histidine residues, nickel-coated agarose beads can be used for purifying the molecules.

The purified molecules obtained are then incubated with the proteins possessing the binding sites for the Fc region.

Advantageously, these proteins are labelled for the purposes of detection, for example with a fluorophore.

When the molecule obtained does not have an antigenic peptide, and we wish to have available antigenic peptide/MHC analogue complexes, it is incubated with the said peptide *in vitro*.

The study of the recombinant molecules according to the invention has demonstrated their great stability, and strong affinity in immunological recognition tests.

The invention thus provides tools that are of considerable interest for modulating immunological processes.

In particular it relates to the use of antigenic peptide/class II MHC analogue complexes for counting and/or purifying the T lymphocytes that react with a given antigen

and for characterizing the phenotype of these cells, i.e. for determining or identifying the molecules that they secrete or that they express on their surface. This detection is carried out on a sample taken from a patient. This can be a blood sample, or a sample taken from secondary lymphoid organs, such as the lymph nodes, the spleen, or from tumours.

These molecules can be used advantageously for counting or for purifying these cells from cellular suspensions as described above.

Alternatively, they can be used for visualization of these cells in cell sections.

It is thus possible to determine the immunological status of an individual.

This application is of considerable interest for the development of vaccines against certain pathogens or of antitumour vaccines.

It is known that for judging the efficacy of a vaccine, the best method is to vaccinate a large number of individuals and to monitor what becomes of this population when it is exposed to the infective agent in natural conditions. However, this approach is difficult, notably because of the considerable costs involved, and the difficulty of finding a sufficient number of volunteers.

The use of complexes according to the invention, as molecular probes that bind selectively to $CD4^+$ T lymphocytes of given specificity, permits rapid comparison of the efficacy of different vaccine preparations and determination of the number and the optimum intervals between boosters.

In a preclinical study, individuals are inoculated with vaccine preparations containing the antigen or antigens, then a count is taken of the T cells present in a sample, that react with complexes according to the invention. The

response of the individuals makes it possible to assess the reaction to the antigenic peptide.

This application can also be employed as predictive means as to a patient's condition, by counting and determining the phenotype of autoreactive T cells in patients at risk.

The invention thus makes it possible to determine the degree of progression of the disease in patients suffering from autoimmune diseases or to evaluate the efficacy of certain treatments or therapeutic interventions.

The invention also relates to the application of the said multivalent complexes defined above in the diagnosis and development of treatments for autoimmune diseases.

A certain number of autoimmune diseases are due to the mobilization of autoreactive T lymphocytes that cause the destruction of the organism's tissues. In some cases, for example in diabetics, the disease is diagnosed late, when the tissues are already destroyed. To prevent the destruction of tissues, and block the development of the disease, it is essential to make an early diagnosis. The possibility of counting, by means of the invention, the autoreactive T lymphocytes in the blood of patients at risk constitutes a considerable advance.

Taking into account that the autoreactive T lymphocytes play a decisive role in the development of autoimmune diseases, very many therapeutic strategies aim to eliminate these lymphocytes, or prevent them exerting their pathogenicity, it can be seen that there is a great advantage in being able to count, by means of the invention, the autoreactive T lymphocytes in the blood of treated patients, to compare the efficacy of different treatments, and to adapt the treatment according to the patient's response.

According to another aspect, the invention relates to the use of the complexes for enrichment in a given type of T cells.

This application makes it possible to have available large quantities of specific T cells of a given antigen *in vitro* for purposes of cellular therapy. The patients can in fact be reinoculated with these cells for prevention or cure. Once again it is possible to count and determine, prior to inoculation, the phenotype of the complexed T cells.

The invention further relates to the application of multivalent recombinant molecules as T-cell-stimulating agents.

An individual can be inoculated with these molecules in order to stimulate the expansion and/or the activation of specific T cells of a given antigen in the absence of any other cell, in particular of presenting cells.

This use is therefore of interest for stimulating inadequate immune responses, for example with respect to MHC/tumour antigen complexes.

In the case of infectious diseases, the recombinant molecules are inoculated *in vivo*, if necessary after a previous stage of propagation *ex vivo*.

Other characteristics and advantages of the invention are given, purely for illustration, in the examples given below and refer to Figures 1 to 5, which show, respectively:

- Figure 1 shows the sequence of the cDNA insert of the Á chain of the MHC,

- Figure 2 shows the plasmid construct containing the cDNA insert of Figure 1,

- Figure 3 shows the sequence of the cDNA insert of the β chain of the MHC,

- Figure 4 shows the plasmid construct containing the cDNA insert of Figure 3,

- Figure 5 shows the detailed plasmid construct of Figure 4, and

- Figure 6 shows a peptide/class II MHC octamer according to the invention.

Example 1: Production of peptide/class II MHC complexes

1. Construction of the recombinant plasmids

. cDNA construct coding for the IAα^d/Fc recombinant protein (clone 461) and insertion in a plasmid

This construct is illustrated by Figure 1 which gives the cDNA sequence, from position 420 to 1940, and that of the coded peptide (437-1921) (SEQ ID No. 1).

The cDNA comprises, linked together successively, the fragments coding for the signal peptide of IA^d, $IA^d\alpha$, a linker, an acidic leucine zipper, a linker, a hinge region, the CH_2 region, then the CH_3 region of Fc.

This construct is inserted in the plasmid shown in Figure 2 and positioned for the control of a $CuSO_4$-inducible metallothionein promoter.

. cDNA construct coding for the recombinant protein LACK/I-A β^d/leucine zipper (clone 268) and insertion in a plasmid

This construct is shown in Figure 3, which gives the cDNA sequence, from position 420 to 1370, and that of the coded peptide (440-1359) (SEQ ID No. 2).

The cDNA comprises successively the fragments, linked together, : coding for a leader sequence, β1, a LACK peptide (158-73), a linker, a thrombin site, a linker, IAβ^d (β1) IAβ^d (β_2), a linker, a basic leucine zipper, a marker with histidine units.

This construct is inserted in the plasmid shown in Figure 4, and shown in detail in Figure 5.

2. Transfection of the plasmids in Drosophila cells

3. Selection of stable transmitters

Stages 2 and 3 are carried out following the procedure according to (6).

4. Production and purification of the complexes

A) Production

The transfected Drosophila cells are cultured in 3-litre bottles, at 24°C, in an SFM Drosophila medium (GIBCO-BRL), supplemented with 1% of FCS (fetal calf serum).

When the cell density reaches 5 x 10^6 cells/ml, the production of LACK/IAd molecules is induced by adding $CuSO_4$ to a final concentration of 1 mM, then the medium is incubated for 5 to 6 days.

The supernatants are combined, and the cell debris is eliminated by centrifugation (20 min, 10K, 4°C). The supernatants are then transferred to tubes and centrifuged again.

The supernatants are concentrated 8 to 10-fold using a Prepscale^R concentrator (Millipore, Inc.). Freezing is effected at -70°C until 500 ml of concentrated supernatants is obtained.

B) Purification

The supernatants are thawed at 37°C. Centrifugation is carried out for 15 minutes at 10K. The supernatants are then transferred to new tubes and are centrifuged again for 15 minutes at 10K.

They are then charged on an MK-D6 immunoaffinity column (bed volume 5 ml), equilibrated beforehand in a buffer A of 20 mM of sodium phosphate pH 7.0. The rate of elution is 10 to 20 ml/h.

The column is washed with 30 ml of buffer A (6 times the volume of the bed) at 0.5 ml/min.

For elution 15 ml of CAPS 50 mM pH 11.5 is used, operating by gravity.

15 fractions, each of 1 ml, are collected.

Each fraction is neutralized with 300 µl of sodium phosphate (200 mM, pH 6.2). Protease inhibitors (Complete^{R}, Roche Diagnostics) are added to each sample immediately.

The column is neutralized with buffer A.

To prevent aggregation of the peptide/MHC molecules, ion exchange chromatography is carried out immediately after elution.

The protein concentration in each fraction is determined by electrophoresis in denaturing gel.

The positive fractions are combined and loaded on an ion exchange column (Mono Q) (Pharmacia Biotech).

A buffer B is used: Tris-HCl 20 mM, pH 8.0, and a buffer C: Tris-HCl 20 mM pH 8.0 + 1 M NaCl.

Operation is effected with the following gradients:

0-5 min: 0% C; 5-20 min: 0-50% C; 20-21 min: 50-100% C; 21-25 min: 100% C; 25-26 min: 100% C; 26-30 min: 0% C.

The LACK/IA^{d} molecules generally elute to 30-36% in buffer C. The fractions corresponding to the elution peak are collected and the protein concentration is determined by electrophoresis in denaturing gel.

The positive fractions are combined and are dialysed at 4°C against 2 l of PBS, pH 7.4.

The dialysis buffer is changed twice in 24 h. The protein concentration is determined by the BCA test (Biorad). The samples are frozen at -70°C in small fractions (8 µg). The yields are of the order of 0.5 mg/l of cellular supernatant.

C. Production of multivalent complexes (Figure 6)

A solution of protein A is prepared, coupled to a fluorophore consisting of Alexa 488^{R} (molecular probes # P-11047) at a concentration of 0.5 mg/ml in PBS 1 X, pH 7.4. (Protein A from Sigma)

100 µl aliquots are prepared and frozen at -20°C.

A peptide/MHC molecule aliquot (8 µg) is thawed and 1.1 µl of protein A coupled to the Alexa fluorophore is added. The mixture is incubated at room temperature for 30 min, then a PBS/BSA (bovine serum albumin) 0.1% mixture is added to give a final volume of 50 µl. 1 µl of mouse serum is added, and the product is used directly as staining reagent.

D. Flow cytofluorimetry

T cells from mouse lymph nodes are purified. 10^6 cells are transferred to a tube and the staining reagent is added. Two hours later, the cells are washed in isotonic buffer and are analysed by flow cytofluorimetry. The frequency of cells reacting with the staining reagent is determined by this method.

REFERENCES

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4. Kozono et al., Nature. 369: 151-153, 1994.

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6. Sambrook et al., Molecular Cloning: Second edition (1989).

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10/048116
PCT/FR 28 JAN 2002

CLAIMS

1/ Soluble recombinant proteins, constituted as a minimum from a dimer that is itself formed from α and β chains of class I or II MHC molecules, comprising at the carboxy-terminal end of one or both chains, all or part of an Fc region of an immunoglobulin, these chains comprising if necessary leucine zippers, characterized in that they are combined in several dimers and in particular in tetramers or in octamers and are complexed with natural or artificial proteins, comprising several binding sites for the constant regions of the immunoglobulins, such as protein A or protein G.

2/ Soluble recombinant proteins according to claim 1, characterized in that they are bound covalently or noncovalently to an antigenic peptide.

3/ Soluble recombinant proteins according to claim 2, characterized in that the antigenic peptide is fixed to the amino-terminal end of the β chain by means of a flexible arm.

4/ Nucleotide sequences having a reading frame corresponding to all or part of a protein according to any one of claims 1 to 3.

5/ Expression vectors, in particular plasmids, characterized in that they have a sequence according to claim 4.

6/ Prokaryotic or eukaryotic cells carrying at least one vector according to claim 5.

7/ Use of proteins according to claim 2 or 3, for counting and/or purifying the T lymphocytes that react with a given antigen and for characterizing the phenotype of these cells.

ART 34 AMDT

8/ Use according to Claim 7, as immunostimulating proteins, in particular for the development of vaccines.

9/ Use according to Claim 7, as a means of predicting a patient's condition, for counting and determining the phenotype of autoreactive T cells in patients at risk, or for therapeutic purposes.

10/ Use according to claim 2 or 3, for the purification and/or enrichment of specific T lymphocytes of a given antigen, either from cell cultures, or from samples taken from a patient.

11/ Populations of T lymphocytes enriched with a given type of T cells, such as those obtained according to claim 10, characterized in that they are intended to be used for the purposes of cellular therapy.

ART 34 AMDT

10/048116
531 Rec'd PCT/ 28 JAN 2002

ABSTRACT OF THE DICLOSURE

The invention concerns soluble recombinant proteins, consisting at least of a dimer which is itself formed by ± and ^{2} molecule chains of MHC class I or II. Said proteins are characterised in that they comprise at the carboxy-terminal end of one or both chains, all or part of a Fc region of immunoglobulin. The invention is applicable to recombinant proteins bound to an antigenic peptide in diagnosis or therapy.

1721-47
CP/CT 59837-1488

Nixon & Vanderhye P.C. (10/99)
(Domestic Non-Assigned/Foreign) Page 1

RULE 63 (37 C.F.R. 1.63)
INVENTORS DECLARATION FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, mailing address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled.

RECOMBINANT PROTEINS AND MOLECULAR COMPLEXES DERIVED FROM THESE PROTEINS, ANALOGOUS TO MOLECULES INVOLVED IN IMMUNE RESPONSES

the specification of which (**check** applicable **box(s)**)

- ☐ is attached hereto
- ☒ was filed on January 28, 2002 as U.S. Application Serial No PCT/FR00/02193 (Atty Dkt. No. 1721-47)
- ☒ was filed as PCT international application No PCT/FR00/02193 on 28 July 2000

and (if applicable to U.S. or PCT application) was amended on October 24, 2001

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose to the Patent Office all information known to me to be material to patentability as defined in 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application.

Priority Foreign Application(s) Application Number	Country	Day/Month/Year Filed
		29 July 1999

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below

Application Number	Date/Month/Year Filed
FR 99/09862	29 July 1999

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below

Prior U.S./PCT Application(s). Application Serial No	Day/Month/Year Filed	Status: patented, pending, abandoned
PCT/FR00/02193	28 July 2000	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And on behalf of the owner(s) hereof, I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively owner's/owners' attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Larry S. Nixon, 25640; Arthur H. Crawford, 25327; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffry H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr., 29366; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178; Robert A. Molan, 29834; B. J. Sadoff, 36663; James D. Berquist, 34776; Updeep S. Gill, 37334; Michael J. Shea, 34725; Donald L. Jackson, 41090; Michelle N. Lester, 32331; Frank P. Presta, 19828; Joseph S. Presta, 35329; Joseph A. Rhoa, 37515; Raymond Y. Mah, 41426; Chris Comuntzis, 31097; Gary T. Tanaka, 43180. I also authorize Nixon & Vanderhye to delete any attorney names/numbers no longer with the firm and to act and rely solely on instructions directly communicated from the person, assignee, attorney, firm, or other organization sending instructions to Nixon & Vanderhye on behalf of the owner(s).

30

1 Inventor's Signature _____ Date February 15 2002
Inventor: Nicolas (first) MI GLAICHENHAUS (last) France (citizenship)
Residence (city) Nice (state/country) FRANCE
Mailing Address 88, boulevard Mantega Righi, Nice, FRANCE
(Zip Code) F-06100

2 Inventor's Signature _____ Date February 15 2002
Inventor: Laurent (first) MI MALHERBE (last) France (citizenship)
Residence (city) Valbonne (state/country) FRANCE
Mailing Address 736, chemin des Ames du Purgatoire Résidence Le Goya, Valbonne, FRANCE
(Zip Code) F-06560

☐ *See attached sheet(s) for additional inventor(s) information!!*

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10/048116
Rec'd PCT/PTO 24 MAY 2002

SEQUENCE LISTING

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Gly Gly Gly Gly Ser Leu Val Pro Arg Gly Ser Gly Gly Gly Gly Ser
    50                  55                  60

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Glu Arg His Phe Val Val Gln Phe Lys Gly Glu Cys Tyr Tyr Thr Asn
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Ala Arg Ser Lys Gly Gly Gly Gly Ser Thr Thr Ala Pro Ser Ala Gln
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